Toll-like Receptor 2 Costimulation Potentiates the Antitumor Efficacy of CAR T Cells

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One Sentence Summary:

The innate immune receptor TLR2, incorporated into CARs, enhanced the efficacy of CAR T cells in eliminating leukemia and solid tumors.

1 **Abstract**

2 Chimeric antigen receptor (CAR) T cell immunotherapies have shown unprecedented

3 success in treating leukemia but limited clinical efficacy in solid tumors. Here, we

4 generated 1928zT2 and m28zT2, targeting CD19 and mesothelin, respectively, by

5 introducing the Toll/interleukin-1 receptor (TIR) domain of Toll-like receptor 2 (TLR2)

6 to 1928z and m28z. T cells expressing 1928zT2 or m28zT2 showed improved

7 expansion, persistency and effector function against CD19$^+$ leukemia or mesothelin$^+$

8 solid tumors respectively in vitro and in vivo. In a patient with relapsed B cell acute

9 lymphoblastic leukemia, a single dose of 5×10^4/kg 1928zT2 T cells resulted in robust

10 expansion and leukemia eradication and led to complete remission. Hence, our results

11 demonstrate that TLR2 signaling can contribute to the efficacy of CAR T cells. Further

12 clinical trials are warranted to establish the safety and efficacy of this approach.

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1 **Introduction**

2   The success of the adoptive transfer of CD19-specific chimeric antigen receptor

3 (CAR) T cells represents a major step in cancer immunotherapy. Both CD3ζ and

4 costimulatory domains [1] are needed to generate effective CARs that provide the

5 signals necessary for full activation, expansion and survival of CAR T cells. The

6 incorporation of costimulatory molecules CD28 [2], 4-1BB [3, 4], OX-40 [5], CD27 [6] and

7 ICOS [7] significantly improve the expansion, cytokine production, in vivo persistence

8 and antitumor efficacy of CAR T cells. Moreover, the efficacy of CAR T cells for

9 solid tumors is substantially poorer than that for leukemia [8, 9]. Given the few

10 costimulatory molecules that have been used in the design of CARs, novel

11 intracellular signals are needed to improve their efficacy so as to effectively treat not

12 only leukemia but also solid tumors.

13   The intracellular signaling domains of CARs mainly derive from the

14 costimulatory receptors expressed on T cells that are critical for their adaptive

15 immune responses. Nevertheless, the innate immune receptor Toll-like receptor 2

16 (TLR2) is highly expressed on activated and memory T cells, and the TLR2 signaling

17 increases T cell expansion and cytokine production [10-13], lowers the activation

18 threshold for costimulatory signals delivered by antigen presenting cells (APCs),

19 facilitates the generation of memory CD8 T cells [14-16], and directly triggers Th1

20 effector functions [17]. In addition, TLR2-mediated signaling can abolish the

21 suppressive capacities of regulatory T (Treg) cells[18]. Furthermore, T cell

22 hyporesponsiveness with impaired IFN-γ and IL-2 production can partially be

1   accounted for by diminished expression of TLR2 on T cells [19]. Strikingly, TLR2

2   ligands induce the regression of established leukemia and solid tumors through

3   compromising the suppressive function of Foxp3^{+} Treg cells and enhancing the

4   cytotoxicity of tumor-specific T cells[20, 21]. TLR2 can form heterodimers with TLR1 or

5   TLR6 to initiate a proinflammatory response [22, 23]. However, TLR2 may also form

6   weak homodimers, which allow it to directly bind to MyD88 [24]. Therefore, we

7   proposed that TLR2 signaling could potentiate the antitumor activity of CAR T cells.

8   To test our hypothesis, we generated CARs targeting CD19 (1928z) and

9   mesothelin (m28z) by linking scFv with CD28 and CD3ζ signaling domains and then

10   generated 1928zT2 or m28zT2 by adding the TIR domain of TLR2 to the 3' end of

11   CD3ζ. We compared the functions of CAR T cells with or without the TLR2 signaling

12   domain and found that the addition of a TLR2 domain led to enhanced antitumor

13   responses for both leukemia and solid tumors. To test the efficacy and safety of TLR2

14   construct in humans, we conducted a pilot trial in a patient with relapsed B cell acute

15   lymphoblastic leukemia (B-ALL) with 1928zT2 T cells and found that a single dose

16   $(5 \times 10^4/kg)$ of 1928zT2 T cells led to complete remission. Our data revealed that the

17   innate immune receptor TLR2, in addition to canonical costimulatory receptors, can

18   improve the antitumor efficacy of CARs for both leukemia and solid tumors.

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20   **Results**

21   *TLR2 enhanced the effector functions of CD19-specific CAR T cells in vitro*

22   The CD19-specific second-generation CARs have generated exciting results in

5

1 clinical trials [25-30]. We used 1928z, a CAR-linking FMC63-scFv, CD28

2 transmembrane and endodomain, and the CD3ζ signaling domain as benchmarks to

3 evaluate TLR2 function in CARs. The TIR domain (amino acid 639-784) of TLR2

4 was appended to the CD3ζ signaling domain. Lentiviral vectors that contained GFP

5 (negative control), 1928z and 1928zT2 (Supplementary Figure 1a) were transfected

6 into T cells to generate GFP T, 1928z T and 1928zT2 T cells, respectively. The

7 transduction efficiencies were measured by the percentages of GFP$^+$ cells

8 (Supplementary Figure 1b). To confirm the role of GFP in representing CAR

9 expression, we detected the coexpression of GFP and anti-CD19 CAR

10 (Supplementary Figure 1c). K562, K562-CD19, NALM6 and REH cells were tagged

11 with both GFP and luciferase (GL). K562-CD19-GL, NALM6-GL, and REH-GL cells,

12 but not K562-GL cells, were specifically killed by 1928z T and 1928zT2 T cells, and

13 notably, the killing percentages of 1928zT2 T cells were higher than those of 1928z T

14 cells at low effector to target (E/T) ratios (Figure 1a). Expression of *GZMB, PRF1* and

15 *IFNG* were higher in 1928zT2 T cells compared to 1928z T cells upon stimulation by

16 NALM6 cells (Supplementary Figure 2). Consistently, 1928zT2 T cells secreted

17 significantly higher amounts of IL-2, IFN-γ and GM-CSF, but a lower level of TNF-α

18 (Figure 1b). Interestingly, suppression of TNF-α secretion from macrophages by

19 TLR2 agonist has been reported [31]. In addition, the capacities of 1928zT2 T cells to

20 expand upon serial stimulations and to continuously eliminate target cells were higher

21 than 1928z T cells (Figure 1c). Moreover, the killing capacity of 1928zT2 T cells was

22 enhanced compared with 1928BBz T cells (Supplementary Figure 3). Taken together,

the incorporation of the TLR2 signaling domain into 1928z led to enhanced

anti-tumor effector functions in vitro.

1928zT2 T cells exhibited enhanced antitumor efficacy in vivo

Next, the killing capacities of 1928z T and 1928zT2 T cells were compared in

cell line-derived xenografts. Immunodeficient NSI mice [32] were intravenously

injected with 1×10^6 NALM6-GL cells followed by two infusions of 5×10^6 GFP T,

1928z T or 1928zT2 T cells. Bioluminescence imaging (BLI) showed that a reduced

tumor burden was present in mice infused with 1928zT2 T cells compared with 1928z

T cells (Figure 2a). Consistently, the percentages of NALM6-GL cells in the

peripheral blood mononuclear cells (PBMC) were significantly lower in mice treated

with 1928zT2 T cells compared with 1928z T cells (Figure 2b). In addition, the mice

treated with 1928zT2 T cells exhibited delayed weight loss (Supplementary Figure

S4a). Furthermore, mice treated with 1928zT2 T cells showed enhanced secretion of

IL-2, IFN-γ and GM-CSF, but not TNF-α, in serum (Supplementary Figure 4b). To

confirm the improved efficacy of the TLR2 construct in terms of survival, 2×10^5

NALM6 cells, and two days later, 2×10^6 GFP T, 1928z T, 1928zT2 T or no T cells,

were intravenously injected into each NSI mice (n=10). 1928zT2 T cells improved the

survival of NALM6 burdened mice better than 1928z T cells (Figure 2c). The

percentages of NALM6 cells and T cells in BM when mice died (Supplementary

Figure 4c) indicated that the major cause of death was high NALM6 burden for mice

treated with no T, GFP T and 1928z T cells, but was graft-versus-host disease (GVHD)

for mice treated with 1928zT2 T cells as severe symptoms such as loss of hair and

weight were observed. Thus, 1928zT2 T cells were more potent than 1928z T cells at

killing NALM6-GL cells in vivo.

Furthermore, the efficacies of 1928z T and 1928zT2 T cells were compared in

patient-derived xenograft (PDX) models. B-ALL cells were eliminated by 1928zT2 T

cells, but not by 1928z T cells, and the percentages of total human T cells and CAR T

cells were higher in 1928zT2 group compared with 1928z group (Supplementary

Figure 5a). Due to the less aggressiveness of primary B-ALL cells, all the B-ALL

burdened mice including the GFP T group survived until when the mice were culled

for analysis on day 78, and splenomegaly was found only in the GFP and 1928z

groups, but not in the 1928zT2 group (Supplementary Figure 5b). Collectively, these

results demonstrated that 1928zT2 T cells had significantly higher anti-leukemia

efficacy than 1928z T cells in vivo.

TLR2 costimulation upregulated genes associated with cell adhesion, synaptic

transmission, T cell migration and mTOR signaling

To decipher the molecular mechanisms of TLR2 costimulation, we performed

RNA sequencing to compare the transcriptomes of 1928z T and 1928zT2 T cells with

or without stimulation by NALM6 cells. Approximately 11013 of the 33382 RefSeq

genes were detectably expressed in all four populations. We identified the genes

specifically upregulated in stimulated 1928zT2 T cells and found that most of these

genes were related to cell adhesion and synaptic transmission (Figure 3a).

Quantitative real-time PCR confirmed the upregulation of genes associated with cell

adhesion and synaptic transmission, along with two genes *IRF3* and *IRF7* downstream

of the TLRs pathway (Figure 3b). TLR2 signaling has been reported to enhance

mTOR signaling and expression of T-bet [33]. Here, we also detected elevated

transcription of *T-bet, mTOR, 4E-BP1* and *P70S6K* (Figure 3b). Matrix

metallopeptidases (MMPs) have been shown to play critical roles in T cell activation

[34] and migration into tissues [35]. TLR2 incorporation also induced the upregulation of

MMP2 and MMP24 expression in 1928zT2 T cells (Figure 3a and 3b). Therefore, the

migration capacities of GFP T, 1928z T and 1928zT2 T cells were compared and

results indicated that 1928zT2 T cells transmigrated the matrigel more efficiently than

1928z T cells (Figure 3c). These results suggest that TLR2 incorporation enhances the

antitumor efficacy of CAR T cells partly through improved adhesion, synaptic

transmission and migration capacities, and enhanced T-bet expression through mTOR

pathway.

TLR2 costimulation enhanced antitumor efficacy of mesothelin-specific CAR T cells

Mesothelin is highly expressed in various solid tumors including lung

adenocarcinoma cells [36], and clinical trials of mesothelin-targeting CAR T cells are

ongoing (such as NCT02414269 and NCT02930993). Thus, to assess the effect of

TLR2 costimulation on CAR T cells targeting solid tumors, we incorporated TLR2

into a CAR specific to mesothelin and generated GFP T, m28z T and m28zT2 T cells

through lentiviral transfection (Supplementary Figure 6a and 6b). m28zT2 T cells

showed enhanced killing capacity against mesothelin[+] A549-GL cells compared with

m28z T cells (Figure 4a). m28zT2 T cells secreted more IL-2, IFN-γ and GM-CSF,

but reduced TNF-α compared with m28z T cells upon stimulation by A549 cells

1 (Figure 4b). The expansion of m28zT2 T cells during repetitive stimulation was

2 improved compared with m28z T cells, and the potency of m28zT2 T cells at killing

3 A549 cells were significantly higher than m28z T cells (Supplementary Figure 7a).

4 Meanwhile the exhaustion markers on T cells after repetitive coculture were

5 compared, and the expression of TIM-3, but not PD-1 or LAG-3, was lower on

6 m28zT2 T cells relative to m28z T cells (Supplementary Figure 7b). These results

7 indicated that TLR2 incorporation in mesothelin-specific CAR also improved its

8 effector functions in vitro.

9 We further compared the antitumor efficacy of m28zT2 T, m28z T and GFP T

10 cells in subcutaneous A549-GL xenografted mice. Tumors from m28zT2 group were

11 smaller than those from the blank, GFP and m28z groups (Figure 4c). Moreover, mice

12 treated with m28zT2 T cells produced more cytokines IL-2, IFN-γ and GM-CSF, but

13 not TNF-α, than those treated with m28z T cells (Supplemental Figure 8). Since

14 metastasis is a major hurdle in the treatment of solid tumors, we compared the

15 efficacies of m28z T and m28zT2 T cells against metastasized tumors through the

16 blood in intravenous A549-GL xenografted mice. The BLI results showed that

17 m28zT2 T cells efficiently inhibited the growth of A549-GL cells in NSI mice;

18 however, surprisingly, the mice grew a greater burden of tumors when treated with

19 m28z T cells than the mice that were injected with GFP T cells (Supplementary Figure

20 9a). The histologic analysis confirmed that the tumor cells in the uterus of the mice

21 from the m28z group were HLA^{+} A549-GL cells (Supplementary Figure 9b). These

22 results indicated that TLR2 incorporation could enhance the efficacy of

1   mesothelin-specific CAR T cells against solid tumors.

2   *Clinical trial of 1928zT2 T cells in a patient with relapsed and refractory B-ALL*

3   We conducted a pilot clinical trial in a patient with relapsed and refractory

4   B-ALL (NCT02822326). 1928zT2 T cells were produced and well characterized

5   phenotypically and functionally (Supplementary Figure 10a-f). The patient received

6   two days of treatment with Fludarabine (30 mg/m2/day) and cyclophosphamide (300

7   mg/m2/day) for lymphocyte depletion, followed by one dose of 1928zT2 T cells at

8   5×10^4 cells/kg (Supplementary Figure 11a). After infusion of 1928zT2 T cells, the

9   patient experienced side effects including fever, coughing, skin rashes and

10 hypotension, but not neurological defects. During this trial, the symptoms of the

11 patients did not necessitate ICU stay, vasopressor support or any steroids treatment. A

12 marked elevation of serum IL-6 was detected on day 2 and tocilizumab, an anti-IL-6R

13 monoclonal antibody [7], was administered at 4 mg/kg, which resulted in rapid

14 defervescence and complete reversal of symptoms over a period of 4 days; however,

15 the patient's concentration of C-reactive protein (CRP) fluctuated in another pattern,

16 and the highest peak on day 45 was due to urological infection (Supplementary Figure

17 11b). In addition, we monitored the dynamic changes of other cytokines

18 (Supplementary Figure 11c). B-ALL cells comprised approximately 15% of the

19 PBMC and more than 70% of the BM before treatment (Figure 5a), but decreased to

20 an undetectable level on day 22 (Figure 5b). Complete remission (CR) was formally

21 diagnosed on day 51 (Figure 5c). Percentage changes of 1928zT2 T cells and CD19^{+}

22 B-ALL cells in PBMC (Figure 5d) and BM (Figure 5e) were depicted. On day 22, the

percentages of $CD4^+CD8^-$, $CD62L^+CD25^-$ and $CD45RO^+CD45RA^-$ populations in

1928zT2 T cells were higher than those in wild-type T cells in both PBMC and BM

(Supplementary Figure 12a). Interestingly, the elevation of $CD4^+$ 1928zT2 T cell

percentage was concomitant with the increase of the percentage of 1928zT2 T cells in

both PBMC and BM (Supplementary Figure 12b and 12c). In addition, the

percentages of $CD25^+$ 1928zT2 T cells were negatively correlated with the

percentages of 1928zT2 T cells in PBMC (Supplementary Figure 12d), while CD62L

expression on 1928zT2 T cells in PBMC remained high until the B-ALL cells were

eradicated (Supplementary Figure 12e). 1928zT2 T cells were detected in

cerebrospinal fluid, indicating the potential role of 1928zT2 T cells in eradicating

leukemia in the central nervous system (Supplementary Figure 12f). The clinical

results here indicated that small dosages of 1928zT2 T cells could kill leukemic cells

with potent efficacy.

Discussion

   Although CD19-specific CAR T cell immunotherapy has achieved promising

results in clinical trials, efficacy of other CAR T cells needs improvement, especially

those for solid tumors. The development of CARs from the first generation (with only

the CD3ζ signaling domain) to the second generation (with CD3ζ and a costimulatory

domain) has revealed a critical role of costimulatory molecules in CARs.

Incorporation of novel costimulatory molecules into CARs should be a feasible

strategy to improve their efficacy.

1   Interestingly, the innate immune receptor TLR2 is expressed on activated T cells as

2   a costimulatory receptor[10]. Innate immunity serves as an immediate defense

3   mechanism against pathogenic organisms. Adaptive immunity plays a delayed but

4   more delicate role in controlling diseases and forming memories. The two types of

5   immunity are not independent of each other and the interaction between them is

6   critical to the outcome of overall immune response. CARs with combinatorial

7   moieties of both adaptive and innate immunity may exert dual functions of mobilizing

8   innate responses as well as promoting T cell expansion, antigen-specific reaction, T

9   cell contraction and memory formation. We demonstrated that 1928zT2 and m28zT2

10   T cells were more potent at killing $CD19^+$ leukemia cells and mesothelin$^+$ lung cancer

11   cells in vitro. TLR2 signaling in CD8 T cells can augment T-bet protein levels and

12   IFN- γ, perforin and granzyme B expression through the mTOR pathway, and

13   contribute to the efficient clearance of intracellular pathogens [33], reminiscent of the

14   results we provide here. To decipher other mechanisms of TLR2-enhanced antitumor

15   efficacy, we found the genes upregulated by TLR2 costimulation, most of which were

16   associated with cell adhesion, synaptic transmission and migration. Therefore, TLR2

17   costimulation can serve as an innate immune moiety to enhance the effector functions

18   and promote adhesion and immune synaptic signaling between CAR T cells and target

19   cells.

20   In our pilot study, only 5×10^4/kg of 1928zT2 T cells expanded to a population that

21   constituted approximately 40% of lymphocytes in patient PBMC, indicating the

22   remarkable expansion capacity of 1928zT2 T cells. Since correlation between infused

1   CAR T cell dose and incidence and severity of cytokine release syndrome (CRS) has

2   been suggested [26, 37, 38], it should be beneficial to reduce the CAR T cell dose. In

3   addition, while large numbers of CAR T cells can be generated, a reduction in CAR-T

4   cell dose may reduce the time for producing enough CAR T cells for therapy and

5   manufacturing cost, and provide capacity advantages, which will be important for

6   widespread adoption of CAR-T cell therapies. Nevertheless, other patients in our

7   clinical trial showed less severe side effects and none needed tocilizumab treatment

8   (data unpublished). Therefore, our TLR2 construct should be potent but without

9   increased incidence and severity of CRS. We provided an unprecedentedly detailed

10   dynamic changes of CAR T cells and B-ALL cells in the patient, a pattern which

11   could be reference for other patients to predict CR. However, results of our ongoing

12   clinical trial will be needed to further determine the safety and efficacy of TLR2

13   co-stimulation in larger patient cohorts.

14   CAR T cells for solid tumors face five major challenges — trafficking, tumor

15   recognition and killing, proliferation and persistence, counteract of microenvironment

16   and control of CAR T cells [39], and the former four of which can be tackled by

17   optimization of costimulations in CARs. In our A549-GL metastatic xenograft mouse

18   models (Supplementary Figure 9a), the tumors in the m28z group were larger than

19   that not only in the m28zT2 group but also in the GFP group. Conversely, we

20   demonstrated that m28z T cells were able to kill A549-GL cells in vitro (Figure 4a)

21   and suppress the growth of A549-GL in subcutaneous xenograft models (Figure 4c).

22   The differences of the results from these experiments show the importance of

1 validating the efficacies of cellular immunotherapies in multiple models. The size of

2 tumors is determined by the combination effects of tumor growth and tumor killing in

3 vivo. It is possible that inflammatory factors secreted by m28z T cells or tumor cells,

4 and cell debris during killing stimulated and recruited mouse tumor-supportive cells,

5 such as MDSCs [40, 41] that easily access to and accumulate in uterus [42, 43]. Then the

6 tumor-supportive cells promoted the expansion of A549-GL cells and overcame the

7 anti-tumor effects of m28z T cells [44]. However, m28zT2 T cells eliminated A549-GL

8 cells efficiently enough so that tumors hardly formed and grew in NSI mice. Of

9 course, further studies were required to test this hypothesis in the future.

10   To summarize, our data demonstrate that TLR2 incorporation can generate more

11 powerful CARs, which have superior anti-tumor efficacies in vitro and in vivo and

12 can be used in patients. These findings reveal an important new strategy for CAR

13 design which bridges the conventional costimulation of adaptive immunity with

14 unconventional innate immune signaling.

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Conflict of Interest Statement

The authors declare no competing financial interests.

Figure legends

Figure 1. 1928zT2 T cells showed enhanced effector functions compared with 1928z T cells in vitro. (**a**) Killing percentages of K562-GL, K562-CD19-GL, NALM6-GL and REH-GL cells by GFP T, 1928z T and 1928zT2 T cells after 18h coculture in vitro. E: T ratios mean the ratios of absolute number of CAR T cells to target cells. The GFP percentages of CAR T cells were equalized by non-transduced T cells from the same donor. Results are representative of at least three independent experiments with T cells from different healthy donors. (**b**) IL-2, IFN-γ, GM-CSF and TNFα

1 concentration in the supernatant of 24h coculture of the indicated T cells with

2 K562-GL or K562-CD19-GL cells (E: T=1, 2×10^5 cells each). (**c**) Enhanced

3 expansion and serial killing potency of 1928zT2 T cells compared with 1928z T cells.

4 1928z T and 1928zT2 T cells were serially co-cultured with NALM6 cells at 1: 4 E/T

5 ratio, the numbers of 1928z T cells and 1928zT2 T cells were counted (left), and

6 percentages of CD19$^+$ NALM6 cells (right) were detected by flow cytometry, every

7 24h for three times. Error bars denote s.e.m and results were compared through

8 two-sided unpaired *t*-test. * P< 0.05, ** P< 0.01, *** P<0.001.

9 **Figure 2.** 1928zT2 T cells showed superior anti-leukemia efficacy in vivo. (**a**)

10 Bioluminescence imaging of NALM6-GL-burdened mice treated with GFP T, 1928z

11 T or 1928zT2 T cells. 1×10^6 NALM6-GL cells were injected intravenously into NSI

12 mice, and on day 2 and 7, 5×10^6 GFP T, 1928z T or 1928zT2 T cells were injected

13 intravenously into each NALM6-GL-NSI mice (n=5). On day 8, 14 and 19,

14 bioluminescence imaging was conducted. (**b**) The percentages of CD19$^+$ (NALM6-GL)

15 cells in the PBMC of mice on day 14. (**c**) Survival analysis of mice treated with GFP

16 T, 1928z T or 1928zT2 T cells (n=10). 2×10^5 NALM6 cells were intravenously

17 injected into NSI mice, and two days later, 2×10^6 T cells were injected (GFP%

18 normalized to 10%), and survival of mice was observed. Error bars denote s.e.m and

19 groups were compared through two-sided unpaired *t*-test. * P< 0.05, ** P< 0.01, ***

20 P<0.001.

21 **Figure 3.** TLR2 costimulation up-regulated genes associated with cell adhesion,

22 synaptic transmission, infiltration and mTOR pathway. (**a**) Heatmap of RNA

1 sequencing results showing genes specifically up-regulated in 1928zT2 T cells after

2 stimulation (+sti) (left). These genes were associated with cell adhesion, synapse

3 components and transmission and cell invasion of T cells (right). (**b**) Relative

4 expression of genes associated with cell adhesion (*CD24, CDH2, TYRO3, CXADR*), T

5 cell infiltration (*MMP2*), synaptic transmission (*CADPS2*), TLRs pathway (*IRF3* and

6 *IRF7*) and mTOR pathway (*T-bet, mTOR, 4E-BP1* and *p70S6K*). (**c**) Transwell cell

7 migration assay indicated an improved capacity of 1928zT2 T cells to degrade and

8 transmigrate Matrigel. T cell culture medium was used as a chemoattractant in the

9 lower chamber. T cells were cultured in the insert with or without coated Matrigel for

10 24 h, and the cells that transmigrated to the lower chamber were counted. The

11 percentage of invasion was calculated as follows: (mean of cells migrating through

12 the Matrigel chamber membrane/mean of cells migrating through the control insert

13 membrane) $\times 100$. Error bars denote s.e.m and groups were compared through

14 two-sided unpaired *t*-test. * P< 0.05, ** P< 0.01, *** P<0.001.

15 **Figure 4.** m28zT2 T cells showed enhanced antitumor efficacy compared with m28z

16 T cells. (**a**) Killing percentages of mesothelin$^+$ A549-GL and mesothelin$^-$ H23-GL

17 cells after 18h coculture with GFP T, m28z T or m28zT2 T cells at the indicated ratios.

18 (**b**) IL-2, IFN-γ, GM-CSF and TNF-α concentration in the supernatant of 24h

19 coculture of GFP T, m28z T or m28zT2 T cells with A549-GL cells. E: T=1:1, 2×10^5

20 cells each. (**c**) Weight of subcutaneous A549-GL tumors from NSI mice treated with

21 no T cells, GFP T, m28z T or m28zT2 T cells. On day 0, 2×10^5A549-GL cells were

22 injected subcutaneously into NSI mice, and on day 7 and 9, no T, 5×10^6 GFP T, m28z

T or m28zT2 T cells were injected through the tail vein into each mouse (n=6). On day 43, mice were sacrificed and tumors were analyzed. Error bars denote s.e.m and groups were compared through two-sided unpaired *t*-test. * P< 0.05, ** P< 0.01, *** P<0.001.

Figure 5. 1928zT2 T cells showed potent efficacy in a patient with relapsed B-ALL. (**a**) FACS analysis of B-ALL cells in PBMC and BM from the patient on the day before infusion of 1928zT2 T cells. (**b**) B-ALL cells were eradicated from PBMC and BM of the patient on day 19 and 22 respectively. (**c**) Wright-Giemsa staining of BM biopsies of the patient on day 18 before infusion (left) and on day 51 post-infusion (right) of 1928zT2 T cells. Scale bar = 10μm. (**d**) Dynamic changes of the percentages of CD19$^+$ cells and 1928zT2 T cells in PBMC of the patient. (**e**) Dynamic changes of the percentages of CD19$^+$ cells and 1928zT2 T cells in BM of the patient.

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